Oestrogen receptor α is required for biochanin A-induced apolipoprotein A-1 mRNA expression in HepG2 cells

Ming Yan Chan¹, Wai Man Gho¹, Zhen-yu Chen¹,², Jun Wang¹ and Lai K. Leung¹,²*
¹Department of Biochemistry, Faculty of Medicine
²Food and Nutritional Sciences Programme, The Chinese University of Hong Kong, Room507C, MMW Bldg, Shatin NT, Hong Kong

(Received 11 October 2006 – Revised 7 March 2007 – Accepted 7 March 2007)

Epidemiological studies have indicated that soya consumption may produce a better plasma lipid profile. The effect may be attributed to the phyto-oestrogens in soya. The red clover (Trifolium pratense) isoflavone biochanin A has a chemical structure similar to those phyto-oestrogens found in soya beans, and is marketed as a nutraceutical for alleviating postmenopausal symptoms. In the present study we investigated the effect of biochanin A on the mRNA expression of ApoA-1 in the hepatic cell line HepG2. Real-time PCR revealed that biochanin A increased ApoA-1 mRNA abundance in cells expressing oestrogen receptor (ER) α. Without ERα transfection, biochanin A had no effect on mRNA abundance. In order to study the transcriptional control, a fragment of the 5’-flanking region of the ApoA-1 gene was amplified and inserted in a firefly luciferase reporter plasmid. The reporter assay indicated that the transactivation of the ApoA-1 promoter was induced by biochanin A in HepG2 cells transfected with the ERα expression plasmid. This induction was reduced by the anti-oestrogen ICI 182,780, whereas the inhibitors of protein kinase (PK) C, PKA, or mitogen-activated kinase (ERK) had no suppressive effect. The present study illustrated that biochanin A might up regulate hepatic apoA-1 mRNA expression through an ER-dependent pathway.

Biochanin A: Oestrogen receptors: Apolipoprotein A-1: Liver cancer cells

CVD comprises the major cause of death in Western countries. Recent projections suggest that CVD will be the leading cause of death in both developed and developing regions of the world by the year 2020¹. Epidemiological studies have associated the consumption of isoflavonoids with a lower incidence of CVD². In normal postmenopausal women, consuming whole soya foods with 60 mg isoflavones per d may help to alleviate several key clinical risk factors for CVD³.

HDL is synthesised in hepatic and intestinal cells and secreted as small particles containing phospholipids, non-esterified cholesterol, ApoA-1 and ApoE. Cholesterol synthesised or deposited in peripheral tissues is returned to the liver in a process referred to as ‘reverse cholesterol transport’. ApoA-1 activates lecithin–cholesterol acyltransferase and facilitates the removal of cholesterol from the tissues (for a review, see Fielding & Fielding⁴).

Oestrogen receptor (ER) α is a member of nuclear hormone receptors for binding a wide range of hydrophobic molecules, such as steroid hormone and phyto-oestrogens. ERα is found in various tissues, including the liver, bone, heart and central nervous system⁵. Oestrogen binds to the C-terminal domain of ERα in the cytoplasm and releases the heat-shock proteins. The activated ERα is translocated into the nucleus and seeks out genes with specific response elements for binding. The gene transcription machinery is then activated and the encoded mRNA is expressed.

Isoflavones are one group of the major phyto-oestrogens that have been the focus of many studies regarding their health benefits. Isoflavones share some common structure with the hormone oestrogen. Despite the similarity, the relative binding affinity of isoflavones for ERα is only 0.05–1 % of the binding affinity of 17β-oestradiol (E₂)⁶. In contrast, their binding affinity for ERβ is approximately seven-fold greater than that of oestrogen. It is suggested that isoflavones may act as selective ER modulators⁷. In addition, the plasma isoflavone concentration can be several thousand times greater than that of E₂⁸. They may compete for ER and display anti-oestrogenic effects, especially when endogenous oestroge level is low. Biochanin A (5,7 dihydroxy-4’-methoxyisoflavone) can be isolated from red clover (Trifolium pratense), and is a nutraceutical for relieving postmenopausal symptoms. The oestrogenic activity of biochanin A is several orders of magnitude lower when compared with other structurally related isoflavones, such as genistein and daidzein⁹.

Previous studies¹⁰–¹² have shown that HepG2 cells can be a viable model for apolipoprotein research except that these cells do not express ERα¹³. By using this cell model, the

Abbreviations: E₂, oestradiol; ER, oestrogen receptor; HNF, hepatocyte nuclear factor; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetra-zolium bromide; PKA, protein kinase A; PKC, protein kinase C.

* Corresponding author: Dr Lai K. Leung, fax + 852 26037732, email laikleung@yahoo.com

The Authors 2007
present study was designed to investigate the regulatory mechanism of biochanin A on ApoA-1.

Materials and methods

Chemicals

Biochanin A was purchased from Sigma Chemicals (St Louis, MO, USA). PD98059, bisindolylmaleimide I and myristoylated PK inhibitor (PKI) 14–22 amide were obtained from EMD Biosciences Inc. (La Jolla, CA, USA). All other chemicals, if not stated, were acquired from Sigma Chemicals.

Cell culture

HepG2 cells (American Tissue Culture Collection, Rockville, MD, USA) were routinely cultured in RPMI-1640 media (Sigma Chemicals), supplemented with 10% fetal bovine serum (Invitrogen Life Technology, Rockville, MD, USA) and antibiotics penicillin (50 U/ml) and streptomycin (50 μg/ml), and incubated at 37°C and 5% carbon dioxide. At 3 days before the experiment, the cultures were switched to RPMI-1640 phenol red-free media (Sigma Chemicals), supplemented with 10% charcoal-dextran-treated fetal bovine serum (Hyclone, UT, USA). The final concentration of the solvent was 0.1% (v/v), and the control cultures received dimethyl sulfoxide only. HepG2 cells were seeded in a six-well plate for 1 day and transfected with ERα expression plasmid or the empty vector pcDNA3-1. The medium was removed and cells were cultured with biochanin A. After 24 h of treatment, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined by absorbance at 260/280 nm. First, DNA strands were synthesised from 3 μg total RNA using oligo-dT primers and Moloney murine leukaemia virus RT (M-MLV RT; USB Corporation, Cleveland, OH, USA). Target fragments were quantified by real-time PCR, and a DNA Engine Opticon™2 (MJ Research, Watertown, MA, USA) was employed for this assay. Taqman®–VIC® MGB probes and primers for apoA-1 and GAPDH (Assay-on-Demand™) and real-time PCR Tagman Universal PCR Master Mix were all obtained from Applied Biosystems (Foster City, CA, USA). PCR reactions were set up as described in the protocol, which was validated by the company. Signals obtained for GAPDH (used as a reference housekeeping gene) were used to normalise the amount of total RNA amplified in each reaction. Relative expression data were analysed using the 2–DDCT method14.

Western analysis

Cells were washed once by PBS (pH 7.4) and harvested into a 1.5 ml microtube with 0.5 ml lysis buffer (PBS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate and 0.1% SDS). The lysis buffer contained protease inhibitors (PMSF (40 mg/l), aprotinin (0.5 mg/l), leupeptin (0.5 mg/l), and pepstatin (0.7 mg/l)). The harvested cells were then lysed with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA) on ice for 30 s. The protein concentration of cell lysate was determined by DC protein assay (BioRad, Richmond, CA, USA). Lysate protein (50 μg) was separated on 10% SDS-PAGE and transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). For the Western analysis performed on culture medium, the volume loaded was normalised with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) absorbance reading. Anti-ApoA-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-actin primary (Sigma Chemicals) and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used for protein detection. An ECL Detection Kit (Amer sham, Arlington Heights, IL, USA) provided the chemiluminescence substrate for horseradish peroxidase, and the targeted protein was visualised by autoradiography.

Measurement of cell viability

Cell number was assessed by MTT staining as described by Mosmann15. Briefly, HepG2 cells were seeded in a ninety-six-well plate and were transfected with ERα and
treated as described earlier. At the end of the treatment, 1500 µl MTT (1 mg/ml) were added to the cells and incubated at 37°C for 4 h. Cell viability was assessed with respect to the absorbance at 544 nm.

**Statistical methods**

A Prism® 3.0 software package (GraphPad Software, Inc., CA, USA) was utilised for statistical analysis. Results of the present study were compared by ANOVA and Bonferroni’s method for multiple comparisons. Significance level was set at $P < 0.05$.

**Results**

**Effect of biochanin A on apolipoprotein A-I mRNA expression**

Cultures transfected with ERα had a dose-dependent increase in ApoA-1 mRNA abundance upon biochanin A treatment, whereas the apoA-1 expression in cells transfected with the empty vector was not affected by the same treatment (Fig. 1). Biochanin A at concentrations of 1 and 10 µM could induce 3- to 5-fold increases in mRNA abundance, whereas E2 at 1 nM elicited an increase of about 7-fold. No induction was observed in empty vector-transfected cultures.

**Response of ApoA-1 promoter to biochanin A in HepG2 cells expressing oestrogen receptor α**

Following the real-time PCR experiment, we carried out reporter gene assays to verify the expression regulation. Significant elevations in the ApoA-1 promoter-driven luciferase activity were demonstrated in ER-transfected cells treated with biochanin A at 0.5 µM or above (Fig. 2), and we observed increased activities ranging from 300 to 600 %. E2 at the concentration of 1 nM also induced a 3-fold increase in the normalised luciferase activities compared with the control.

**Effect of isoflavone on apolipoprotein A-1 protein**

ApoA-1 expression at the protein level was also assessed in cell lysates and culture medium. However, the ApoA-1 protein in cell lysates or culture medium was not increased by biochanin A treatment (Fig. 3). It was possible that the translational machinery was unable to cope with the increased messages in this cell system.
Effect of protein kinase inhibitors and anti-oestrogen on ApoA-1 promoter-driven luciferase activities in cultures expressing oestrogen receptor α

Previous studies\textsuperscript{16,17} have indicated the involvement of protein kinase A (PKA), protein kinase C (PKC) or mitogen-activated protein kinase in ApoA-I regulation. Protein kinase inhibitor (PKI) 14–22 amide, bisindolylmaleimide I and PD98059 are specific inhibitors for PKA, PKC and mitogen-activated protein kinase, respectively. The inhibitor concentrations have been validated in HepG2 cells\textsuperscript{16}. Compared with the control the administration of these inhibitors did not substantially decrease the ApoA-1 promoter-driven gene transactivation (Fig. 4(A)). PD98059 could induce rather than suppress the promoter activity. When the pure anti-oestrogen ICI 182,730 was administered, luciferase activity was significantly ($P < 0.05$) reduced as demonstrated in Fig. 4(B). These data illustrated that ER\textsubscript{α} was involved in biochanin A-induced ApoA-1 transcriptional activation.

Determination of ApoA-1 promoter-driven luciferase activities in oestrogen receptor α-transfected HepG2 cells

HepG2 cells were transfected with various ApoA-I reporter constructs and ER\textsubscript{α} expression plasmid. Luciferase activity was subsequently measured to reveal the transcriptional control of apoA-I expression. All ApoA-I constructs displayed increasing trends when treated with increased concentrations of biochanin A (Fig. 5(A)). The greatest response was observed in the pTA-ApoA-I-luc construct. However, the increases of the luciferase signals were similar among different biochanin A concentrations within the same construct in terms of the percentage induced. This indicated that no critical promoter segment was induced by biochanin A (Fig. 5(B)).

Investigation of ApoA-1 promoter-driven luciferase activities in HepG2 cells expressing oestrogen receptor β

Since ER\textsubscript{β} might also activate oestrogen response element, we carried out another reporter gene assay to determine whether
the expression was also regulated by this ER isoform. Biochanin A at 10 μM showed a marginal elevation in the normalised luciferase activities (Fig. 6), and the increased activities were not deviated from the empty vector. The ApoA-1 promoter-driven luciferase activity was not significantly increased by biochanin A in cells expressing ERβ.

Discussion

In the present study, we found that biochanin A up regulated apoA-1 mRNA expression in HepG2 cells expressing ERα, not ERβ. ApoA-1 promoter-driven reporter gene assays supported that the up regulation was introduced by increased transcriptional activities. The induction pathway appeared to be independent of mitogen-activated kinase (ERK), PKA and PKC. Luciferase assays using the truncation reporter plasmids also did not reveal any critical elements lying between -40 and -2068 in the 5′-flanking region of the ApoA-1 promoter.

17β-E2 and genistein have been shown to increase the promoter activities of ApoA-1 in HepG2 cells18,19. Similarly, the present study demonstrated that biochanin A activated both the ApoA-1 mRNA expression and ApoA-1 promoter activity. The phyto-oestrogen biochanin A appeared to activate ERα for the induction of mRNA expression of ApoA-1, and the condition has not been established in the above-mentioned studies. It has been shown that the mitogen-activated kinase (ERK) activation pathway is increased in the up regulation of ApoA-1 gene expression by genistein and E2 in wild-type HepG2 cells16. Conversely, Beers et al.17 have shown that overexpressing ERK1/2 suppresses rather than encourages the transcriptional activities. The present study indicated that inhibition of several signalling pathways including the mitogen-activated protein kinase, PKC and PKA pathways did not abolish the augmented ApoA-1 transcription. Mitogen-activated protein kinase inhibitor by itself might even increase the transactivity, which could be contradictory to the above-mentioned observations. This suggested that the up regulation of ApoA-1 transcriptional activity in the presence of ERα was probably not going through these signalling pathways.

The first 256 bp upstream in the 5′-flanking region is critical in ApoA-1 regulation in HepG2 cells. Unlike the intestinal Caco-2 cells which require the segment -192 to -2052 for transcriptions, the segment between -41 and -256 is sufficient and specific for maximal ApoA-1 transcription in HepG2 cells20. Previous studies have shown that the increase in ApoA-1 gene expression by oestrogen and genistein is mediated through the -256 to -41 region of the ApoA-1 promoter21,22. This region contains binding sites for three transcription factors, which are hepatocyte nuclear factors (HNF)-3β, HNF-4 and early response factor Egr-1. Sites at -214 to -192 and -169 to -146 have been shown to contain response elements for HNF-423 and HNF-3β22, respectively. Binding sites for Egr-1 have also been located at -221 to -231 and -189 to -18124. Our findings did not support the notion that oestrogen increased ApoA-1 mRNA expression in HepG2 cells without ERα. With respect to our truncation reporter experiments performed in HepG2 cells expressing ERα, biochanin A treatment did not initiate a higher luciferase response than the control in cells transfected with reporter plasmids constructed by deleting sequences from -2068 to -40. Hence, the cis-acting DNA binding sites activated by biochanin A was apparently not present in this segment. Because an increase in promoter activity was observed in the reporter assays, biochanin A might activate an enhancer element or deactivate a repressor element in a region further upstream or downstream of the gene. A 48 bp enhancer element located at the far distal region of ApoA-1 (+8446/+8399) has been identified24, which could be a possible activation pathway in the present study.

Oestrogen replacement therapy has long been used for controlling postmenopausal symptoms, including lowering blood cholesterol. Lamon-Fava et al.18 have demonstrated that E2 increases promoter activities of ApoA-1 in HepG2 cells. An ER-independent pathway has also been described by Zhang et al.25 for equine oestrogen in the up regulation of ApoA-1 promoter activity. In contrast, oestrogen may repress ApoA-1 expression. Harnish et al.15 observed that 100 nM-E2 represses ApoA-1 promoter activity in HepG2 cells stably expressing ERα. These contradictory observations can be explained by differences in the oestrogen concentration, timing, or the model nature.

Acknowledgements

The present study was supported by the Chinese University of Hong Kong Direct Grant for Research (code no. 2041184).

References


